# Ameliorating effects of mesenchymal stem cells on testicular injury in a prepubescent male rat model of streptozotocin-induced diabetes

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## ABSTRACT

The oxidative stress, inflammation and apoptosis contribute to testicular dysfunction in patients with diabetes mellitus (DM). Accordingly, the present study aimed to evaluate the potential ameliorative effects of systemic administration of bone marrow mesenchymal stem cells (BM-MSCs) on testicular injury in prepubescent male rats with streptozotocin (STZ)-induced diabetes. A total of 24 four-week-old male albino rats were equally divided into four groups: rats administered normal saline only (control group), STZ-treated rats (diabetes group), STZ-treated rats administered $2\times10^6$ BM-MSCs one week after STZ therapy (early MSC treatment group), and rats administered $2\times10^6$ BM-MSCs four weeks after STZ injection (late MSC treatment group). At seven days after BM-MSC administration, serum and testicular tissue samples were obtained for biochemical, histological, and ultrastructural analyses. The results indicated that in contrast to the control group, diabetes group showed low levels of testosterone, follicle-stimulating hormone, and luteinizing hormone in sera, and decreased level of glutathione and diminished activities of superoxide dismutase, catalase, and glutathione peroxidase in testicular tissues. However, the total oxidant status, thiobarbituric acid reactive substances, tumor necrosis factor-α and interleukin-1β were significantly increased in diabetes group compared to the control one. Also, the histological examination demonstrated hypoplasia and arrest of spermatogenesis at various stages in diabetes group. Moreover, the cytoplasm of their spermatogenic cells was characterized by a high number of defective mitochondria and expanded rough endoplasmic reticula. On the other hand, the early administration of BM-MSCs ameliorated most of the effects of STZ-induced diabetes in testicular tissues. These findings demonstrate that early systemic administration of BM-MSCs to prepubescent rats can protect against testicular injury and may restore testicular function in a model of STZ-induced diabetes.

*Keywords*: BM-MSCs, testis, oxidative stress, Pro-inflammatory cytokines.

## INTRODUCTION

Diabetes mellitus (DM) is a progressive metabolic disorder associated with morbidity and mortality of rates (Roglic, 2016; Shi *et al*., 2018). DM has delirious effects on many organs, including the testis, and contributes to the development of multiple metabolic syndromes (Barky *et al*., 2017; Ding *et al*., 2015). Type 2 DM-associated infertility has been reported in men of reproductive age including teenagers (Heidari Khoei *et al*., 2019). Prepuberty is a critical time in the development of adult fertility in rats (Moody *et al*., 2013).

Stem cell technology included cell replacement therapy has progressed rapidly in the last few decades (Millman and Pagliuca, 2017). Mesenchymal stem cells (MSCs) are regenerating cell types with potential clinical utility for the treatment of a variety of disorders (Caplan and Dennis,

2006). Transplanted MSCs have the ability to migrate to sites of injury and differentiate into functional cell types specific to the target organ (Cao *et al.*, 2016). MSCs have been reported to reverse testicular injury in rats treated with busulfan (Mehrabani *et al.*, 2015; Qian *et al.*, 2020). A recent review by Hoang *et al.* (2022) posited that MSCs represent potential candidates for the treatment of a wide range of clinical conditions including bone marrow mesenchymal stem cells (BM-MSCs) for brain and spinal cord injuries, adipose tissue MSCs for reproductive disorders and skin regeneration, and umbilical cord MSCs for treating a wide human diseases.

Increased understanding of basic stem cell pathways may facilitate the further development of stem cell-based regenerative medicine and treatment for diabetes. Accordingly, the present study aimed to assess the potential ameliorative effects of systemic injection of BM-MSCs on the testis of prepubescent male rats in a streptozotocin (STZ)-induced model of diabetes.

## MATERIALS AND METHODS
### Experimental animals

Twenty-four healthy prepubescent male albino rats (*Rattus norvegicus*) at approximately 4 weeks of age weighing 60–80 g were obtained from the Egyptian Organization for Biological Products and Vaccines, Giza, Egypt. Rats were housed in clean plastic cages with wood chips and provided a conventional rat pellet diet and water *ad libitum*. Environmental conditions were maintained at room temperature (25±2°C) with a 12/12 hour light-dark cycle and a relative humidity of 55±5%. All rats were acclimatized for one week prior to the experimental period. Animals were tagged and body weights were recorded at the beginning of the experiment. The present study adhered to the international standards for animal laboratory treatment and the guidelines of Ain Shams University's Institutional Animal Ethics Committee for the use and treatment of animals.

### Induction of experimental diabetes

Prepubescent rats fasted for 12 hours were administered a single intraperitoneal (IP) injection of freshly formulated STZ (Sigma-Aldrich, St. Louis, MO, USA) at a dose of 45 mg/kg of body weight dissolved in ice-cold saline (pH, 4.5) in a volume of 1 ml/kg body weight (Patil *et al.*, 2011). To reduce the risk of hypoglycemia and death, rats were allowed to drink 5% glucose solution *ad libitum* overnight after STZ administration. Venous blood samples were drawn from the tail vein 72 hours after STZ administration and used to measure fasting blood glucose (FBG) levels using a one touch ultra-glucometer and compatible blood glucose strips. Rats with FBG levels $\geq 250$ mg/dl were considered diabetic and used in subsequent experiments. Control rats were administered normal saline solution at the same time as STZ-treated rats.

### Isolation and culture of bone marrow mesenchymal stem cells

Bone marrow was extracted from ten six-week-old adult male white albino rats. After sedation with mild ether anesthesia, femurs and tibiae were dissected, and muscle and connective tissues were removed. The proximal and distal ends of bones were removed and marrow cavities were flushed with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO/BRL) to harvest bone marrow. Ten milliliters aliquots of bone marrow suspension were carefully layered over 5 ml of Lymphoprep (Stemcell Technologies Inc., Vancouver, Canada) in a 50-ml centrifuge tube and then centrifuged at 800 ×g for 20 minutes at room temperature. The density gradient centrifugation method was used to sort cells according to size and density. Nucleated cells were isolated using an interface gradient (Ficoll/Paque, Pharmacia). Isolated

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cells were resuspended in complete culture medium enriched with 1% penicillin–streptomycin (GIBCO/BRL) and cultured at 37°C for 12–14 days in 5% humidified $CO_2$. Once large colonies have formed (80–90% confluence), cultures were washed twice with phosphate buffer saline (pH,7) and trypsinized at 37°C for 5 minutes with 0.25% trypsin in 1 mM EDTA. After centrifugation, cells were resuspended in serum-augmented media and incubated in 50 $cm^3$ culture flasks (Falcon). For flow cytometry, 300 ml cell suspensions were incubated for 45 minutes at room temperature in the presence of antibodies directed against CD29, CD105, and CD34 and CD90 antibodies conjugated with allophycocyanin (APC), cyanine 5 (CY5), phycoerythrin (PE), or fluorescein isothiocyanate (FITC). Flow cytometry was performed using a FACS Calibur (BD Biosciences, Germany). Data were analyzed in Cell Quest software. Cultures resulting from cells isolated by cell sorting were considered first-passage cultures in accordance with Alhadlaq and Mao (Alhadlaq and Mao, 2004). MSCs were characterized by fusiform appearance and surface adhesion (Jaiswal *et al*., 1997).

## Experimental design

Rats were equally divided into four groups each containing six rats. Figure (1) displays the study's general layout.

**a-The control group** comprised male rats administered normal saline solution according to the same methods used for MSC administration.

**b-The diabetes group** comprised male rats administered STZ.

c-T**he early MSC treatment group** comprised STZ-treated rats administered $2 \times 10^6$ BM-MSCs in a 0.5 ml volume into the caudal vein (Antunes *et al*., 2014). This dosage was administered during the early phase of diabetes induction (one week after STZ administration).

d-**The late MSC treatment group** comprised STZ-treated rats administered $2 \times 10^6$ BM-MSCs in a 0.5 ml volume into the caudal vein during the late phase of diabetes induction (4 weeks after STZ administration).

## Collection of sera and tissue samples

After seven days of MSC administered, animals were fasted overnight and then sedated with isoflurane anesthesia. Cardiac puncture was used to collect blood samples. Blood samples were centrifuged at 1500 g for 10 minutes at 4°C. Obtained sera were immediately frozen and stored at −80°C until further use. Testicular tissue samples were also harvested from rats in all groups for biochemical, histological, and ultrastructural analyses.

## Preparation of testicular tissue homogenates

Harvested testes were sliced, weighed, and homogenized in 50 mmol ice-cold phosphate buffer to obtain a 10% solution (w/v). Homogenates were centrifuged for 15 minutes at 4000 rpm and 4°C to remove debris. Clear supernatants were separated and stored at −80°C until use for protein analysis and biochemical tests. Testicular protein content was measured according to the method of Lowry *et al*. (1951) using bovine serum albumin as a standard.

## Biochemical analyses

Serum concentrations of testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) were determined using enzyme-linked immunosorbent assay (ELISA) kits in accordance with the manufacturer's instructions.

Markers of oxidative stress in testicular supernatants were determined using well-established techniques. The

obtained values were adjusted for the protein content of each sample. The level of lipid peroxidation was measured as the content of thiobarbituric acid reactive substance (TBARS) according to the method of Ohkawa *et al.* (1979), with the results expressed as nmol TBARS/mg protein. The total oxidant status (TOS) was evaluated using a spectrophotometer by determining the oxidation of ferrous ion-chelate complexes to ferric ions by the amount of oxidants present in tissue samples (Erel, 2005), with the results expressed as nmol $H_2O_2$ Equiv/mg protein.

Commercially available colorimetric kits (Biodiagnostic, Egypt) and a UV-vis spectrophotometer (Shimadzu, Kyoto, Japan) were used to measure the activities of antioxidant enzymes in testicular tissues including superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), and glutathione peroxidase (GPx). SOD activity measured evaluated using the procedure of Sun *et al.* (1988). CAT activity was measured using the Aebi approach (Aebi, 1984) by measuring $H_2O_2$ consumption. The activities of GSH and GPx were measured using the methods described by Moron *et al.* (1979) and Paglia and Valentine (1967), respectively.

The levels of two proinflammatory cytokines, tumor necrosis factor-alpha (TNF-α) and Interleukin 1β (IL-1β), in testicular tissues were measured using commercially available ELISA kits (Biodiagnostic, Egypt) as previously reported by Kumar *et al.* (2018).

## Histological and ultrastructural analyses

Small testicular fragments were immediately immersed in aqueous Bouin's solution for 24 hours. Paraffin-embedded slices (5 μm thickness) were stained with hematoxylin and eosin (Bancroft and Gamble, 2002) and imaged under light microscopy.

For electron microscopy (Dykstra *et al.*, 2002), freshly removed testicular tissue samples were cut into extremely small pieces and fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 mol cacodylate buffer (pH, 7.4) for 4 hours. Samples were then post-fixed in a buffered solution of 1% osmium tetroxide at 4°C for 1–5 hours. This was followed by dehydration in ascending concentrations of ethyl alcohol, clearing in propylene oxide with two 5-minute changes, and embedding in EPON epoxy resin. Toluidine blue was used to stain semi-thin sections of 1 μm thickness which were then examined using a bright field light microscope. Sections were trimmed to be ultrathin, mounted on form var-coated grids, and stained with uranyl acetate and lead citrate (Reynolds, 1963). Sections were inspected and photographed using a Joel transmission electron microscope (JEOL Inc., Peabody, MA, USA) at Al-Azhar University's Regional Center for Mycology and Biotechnology (RCMB), Egypt.

Two sections from each MSC treatment group were immunostained with anti-Brdu and goat anti-mouse Ig and then visualized under a fluorescent microscope to detect the presence of MSCs in testicular tissues (Fig. 2).

## Statistical analyses

Data were analyzed using IBM SPSS Statistics for Windows, version 22 (IBM Corp., Armonk, New York, United States). Data are presented as the mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) with Tukey's post-hoc test was used to compare statistical differences between treatment groups. P-values less than 0.05 were considered statistically significant.

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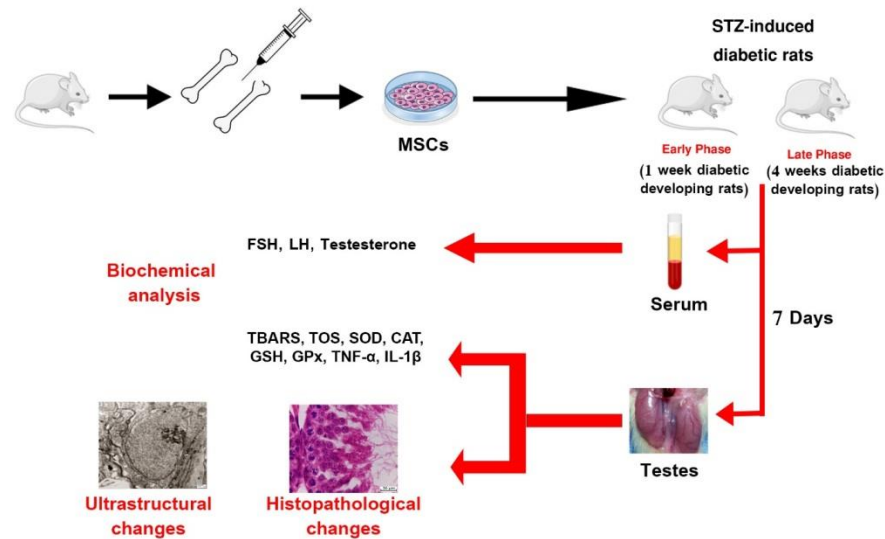


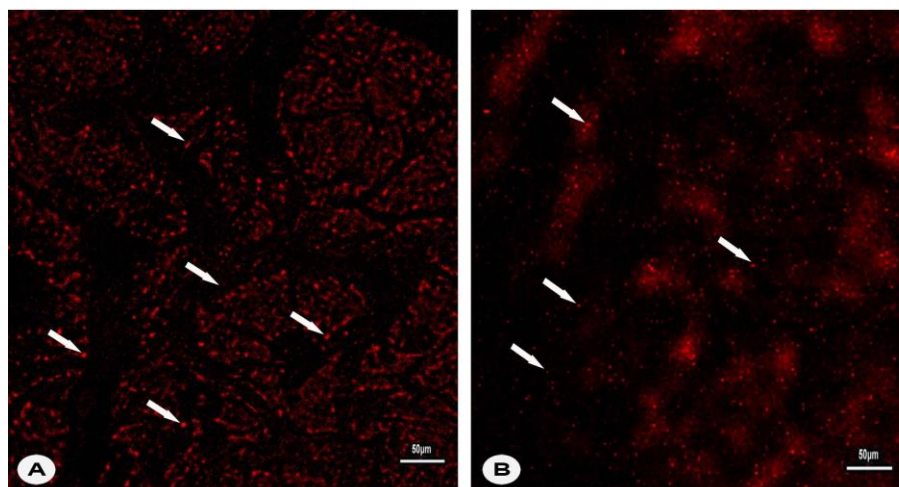**Fig. 1.** Illustration of experimental design and groups with measured parameters.



**Fig. 2.** Fluorescent-labeled BM-MSCs immunostained with 5-bromo-2'-deoxyuridine (BrdU) in testicular tissues from STZ-treated rats in the early MSC treatment group (A) and late MSC treatment group (B).

## RESULTS

### Biochemical analyses

To assess testicular injury, we measured serum levels of sex hormones (testosterone, FSH, and LH), testicular antioxidant activity (TBARS, TOS, SOD, CAT, GSH, and GPx), and inflammatory biomarkers (IL-6 and TNF-α).

As illustrated in Fig. 3, significantly lower serum LH, FSH, and testosterone levels ($P \leq 0.05$ for all) were observed in STZ-treated rats compared to the control group. Although serum levels of sex hormones were slightly higher in the late MSCs treatment group compared to STZ-treated rats, a significant difference remained in serum LH, FSH, and testosterone levels between the late MSCs treatment and control groups ($P \leq 0.05$ for all). Early treatment with MSCs led to

increased serum levels of LH, FSH, and testosterone compared to the diabetes group (P ≤ 0.05 for all).

To assess the possible effects of intravenous MSCs administration on markers of oxidative stress in testicular tissues, the levels of TBARS and the activities of TOS, SOD, CAT, GSH, and GPx were measured in testicular homogenates from prepubescent male rats in the control and treatment groups. It was found that there was an increasing in TBARS levels and TOS activity with significantly decreasing activity of SOD, CAT, GSH, and GPx in STZ-treated rats compared to the control group. Although levels of oxidative stress markers were slightly higher in the late MSCs treatment group compared to the diabetes group, a significant difference was observed between the late MSCs treatment and control groups (P ≤ 0.05). In the early MSCs treatment group, TOS, SOD, CAT, GSH, and GPx activities were almost completely restored to

the levels observed in the control group. Although partial ameliorated TBARS content was observed in the early MSCs treatment group, a significant difference remained between the early MSCs treatment and the control groups (P ≤ 0.05).

The effect of BM-MSCs administration on STZ-mediated testicular inflammation is shown in Figure (5). STZ treatment stimulated an inflammatory response with increased testicular levels of TNF-α and IL-1β compared to the control group (P ≤ 0.05 for both). Conversely, early BM-MSCs treatment reduced testicular inflammation in prepubescent diabetic male rats, as demonstrated by a significant decline in serum TNF-α and IL-1β levels compared to the diabetes group (P ≤ 0.05). Late treatment with BM-MSCs during the late phase resulted in small changes in these parameters compared to the diabetes group; however, the difference between the late MSCs treatment and control groups remained statistically significant (P ≤ 0.05).
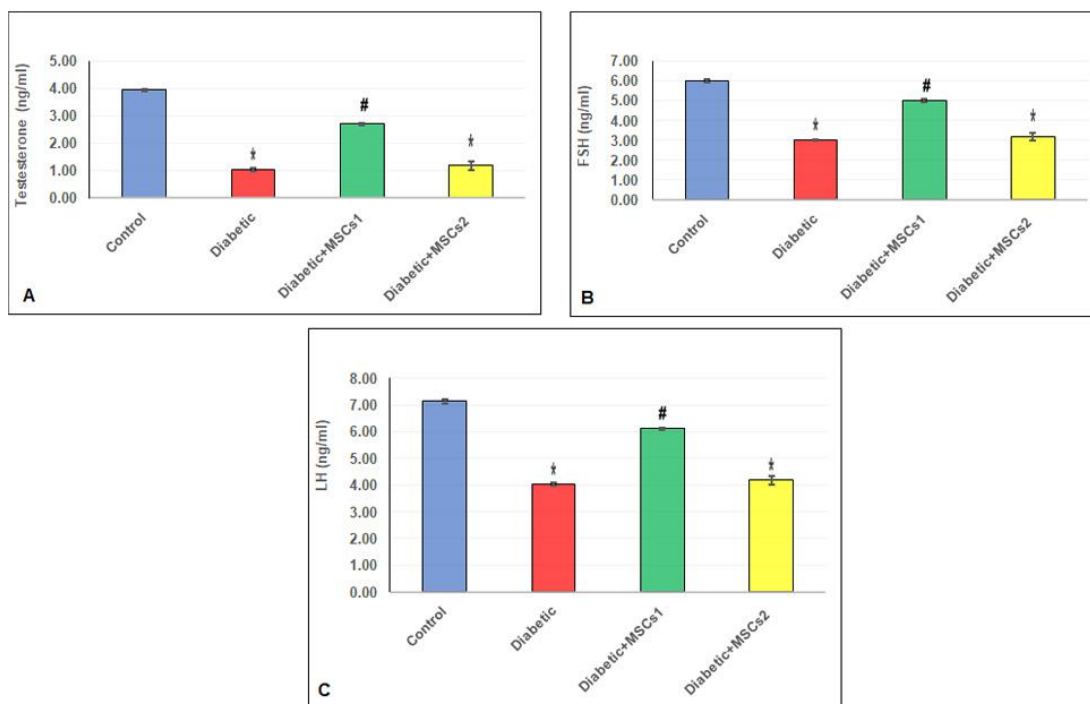


**Fig. 3.** Effect of systemic bone marrow mesenchymal stem cells administration on serum levels of (a) testosterone, (b) FSH, and (c) LH in the control and treatment groups of prepubescent male rats. Values are presented as mean ± SEM (n = 6). ∗, P ≤ 0.05 versus the control group; #, P ≤ 0.05 versus the diabetes group.

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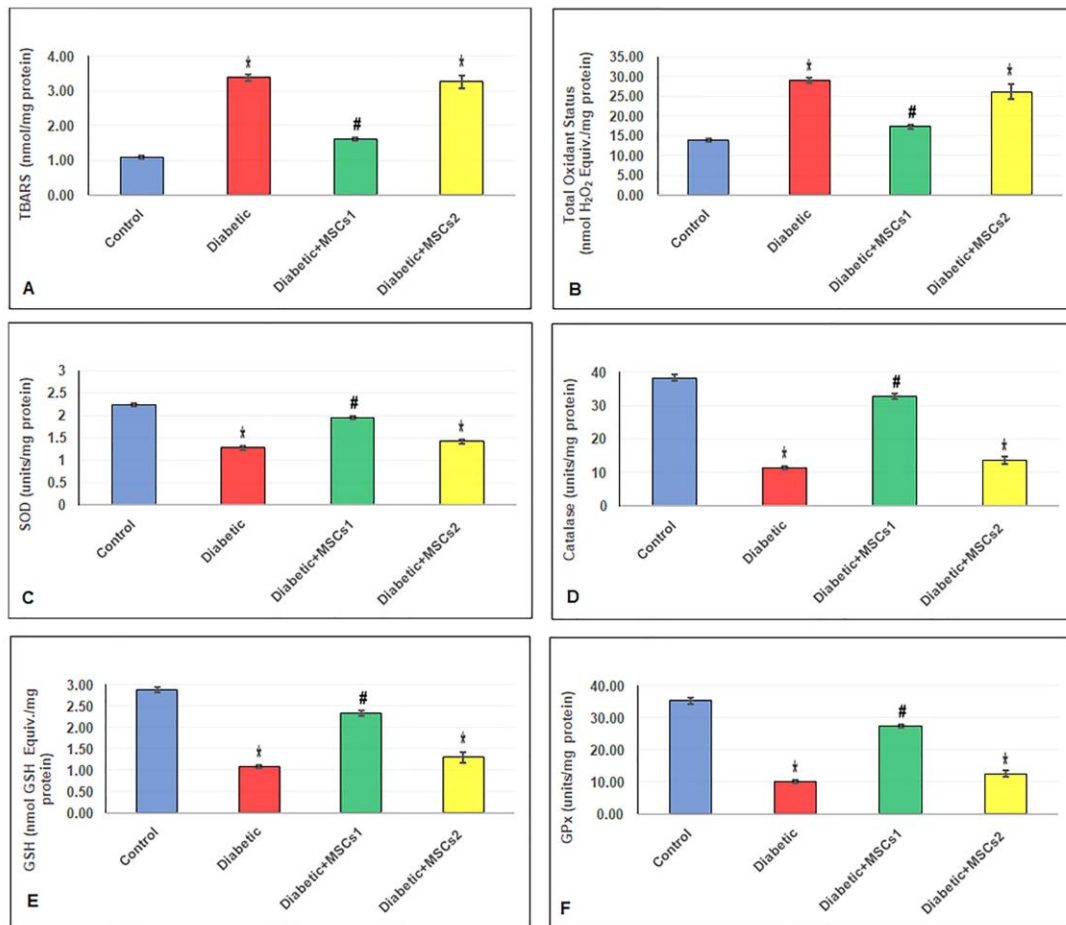


**Fig. 4.** Effect of systemic bone marrow mesenchymal stem cells administration on markers of oxidative stress in testicular tissues of prepubescent rats in the control and treatment groups. **(a),** Thiobarbituric acid reactive substance (TBARS); **(b),** total oxidant status (TOS); **(c),** superoxide dismutase (SOD); **(d),** catalase (CAT); **(e),** glutathione (GSH); and **(f),** glutathione peroxidase (GPx). Values are presented as mean ± SEM (n = 6). ∗, P ≤ 0.05 versus the control group; #, P ≤ 0.05 versus the diabetes group.
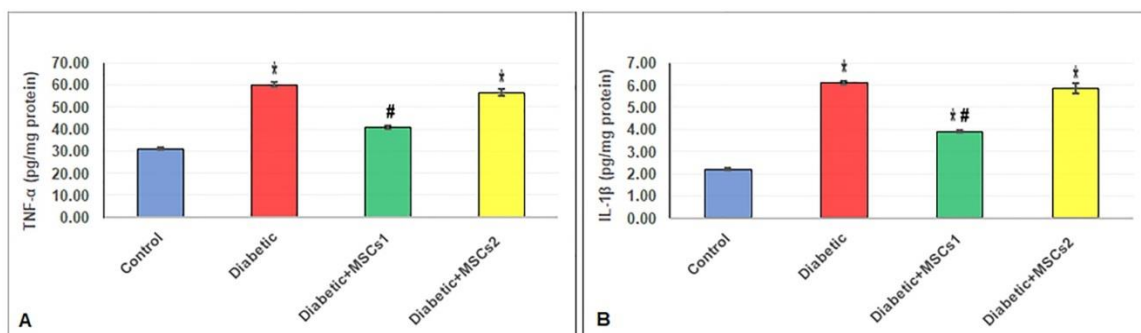


**Fig. 5.** Effect of systemic bone marrow mesenchymal stem cells administration on biomarkers of inflammation in testicular tissues of prepubescent male rats in the control and treatment groups. **(a),** Tumor necrosis factor-alpha (TNF-α); **(b),** interleukin 1β (IL-1β). Values are presented as mean ± SEM (n = 6). ∗, P ≤ 0.05 versus the control group; #, P ≤ 0.05 versus the diabetes group.

## Histological and histopathological observations

Histological examination of testicular tissue from control prepubescent male rats demonstrated normal histological structure. A thick fibrous tissue capsule, known as the tunica albuginea, surrounds the rate testis. The testis is partitioned into lobules by thin fibrous septa with each lobule comprising multiple seminiferous tubules bordered by interstitial tissue. Leydig cells were observed in the surrounding interstitium as single cells or as clumps embedded in the rich plexus of blood lymph capillaries surrounding the seminiferous tubules (Fig. 6a). Each tubule was lined by germ cells in various stages of spermatogenesis, with Sertoli cells observed in between. The spermatogenic lineage is composed of spermatogonia, primary and secondary spermatocytes, spermatids, and mature spermatozoa that occupy the center of the lobule. Sertoli cells nourish the developing spermatozoa and were observed between the spermatogonia and at the basal lamina (Fig. 6b).

Histological examination of testicular tissue from STZ-treated rats demonstrated sloughed seminiferous tubules which were detached from each other creating widening of intertubular spaces (Fig. 6c). These findings are indicative of degradation of the interstitial tissue and Leydig cells which exhibited visible degeneration and hypoplasia with only the remnants of a few Leydig cells observed between seminiferous tubules. Congestion of blood vessels and interstitial edema were observed in interstitial tissue (Fig. 6d).

On the contrary, testicular tissue from the early MSCs treatment group demonstrated restoration of most germinal cells and spermatozoa with several layers of spermatogenic cells compared to testicular tissue from STZ-treated rats. A normal configuration of seminiferous tubules and Leydig cells was also observed (Fig. 6e,f).

Similar histopathological features were observed in testicular tissues from the late MSCs treatment group and STZ-treated. Moreover, arrest of spermatogenesis at various stages was observed with some seminiferous tubules devoid of spermatozoa (azoospermia) in the late MSCs treatment group (Fig. 6g,h).

## Ultrastructural observations

Electron microscopy studies of testicular tissues from the control group of prepubescent male rats demonstrated that each seminiferous tubule was surrounded by a thin basal lamina with alternating boundary layers, each one consisting of an inner thin layer of collagenous fibers surrounded by outer spindle-shaped smooth muscle cells (myoid cells). Sertoli cells rest on the basal lamina of the seminiferous tubules and were observed with irregular shape and cytoplasm extending to the lumen of the tubule to full the narrow spaces between the cells of the spermatogenic series. The cytoplasm contained abundant endoplasmic reticulum, a moderate number of mitochondria, free ribosomes, and a number of lysosomes. Nuclei were polymorphous with deep indentations and possessed a prominent nucleolus (Fig. 7a). Spermatogonia were observed at the basal lamina. Mitochondria, rough endoplasmic reticulum, and free ribosomes were observed in the cytoplasm of spermatogonia (Fig. 8a). Primary spermatocytes were observed as large cells with spherical nuclei. The cytoplasm of primary spermatocytes contained oval mitochondria with many free ribosomes and small cisternae of the endoplasmic reticulum (Fig. 8b). Spermatids were observed as small spherical cells with spherical nuclei close to the lumen of seminiferous tubules. The cytoplasm of spermatids contained endoplasmic reticulum with flattened cisternae, ribosomes, and mitochondria with vacuolated appearance

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(Fig. 8c). Through the spermatogenesis process, the spermatids undergo metamorphosis into spermatozoa. Leydig cells in tissues from the control groups were relatively large and possessed large spherical nuclei with distinct nucleoli. The cytoplasm contained lipid droplets and abundant smooth endoplasmic reticulum in the form of a network of interconnecting tubules. Mitochondria were abundant and varied in size and shape. The cisternae of the rough endoplasmic reticulum were observed as small groups parallel to each other (Fig. 9a).

At the ultrastructural level, testicular tissues from diabetic rats had irregularity and thickening of the basal laminae of seminiferous tubules. Sertoli cells had scattered aberrant mitochondria and irregularly shaped nuclei (Fig. 7b). Most spermatogenic cells had altered structures with vacuolated mitochondria observed as almost empty sacs with no internal configuration. Clumps of heterochromatin were observed on the inner surface of the nuclear envelope in spermatogonia nuclei. Spermatids were poorly developed with cytoplasm containing vacuolated mitochondria and fragmented rough endoplasmic reticulum (Fig. 8d,e,f). Multiple lipid droplets and scattered rough endoplasmic reticula were the most prominent cytoplasmic alterations observed in Leydig cells, with clumps of highly condensed chromatin observed on the internal surface of the nuclear envelope (Fig. 9b).

Electron microscopy of testicular tissue from rats in the early MSCs treatment group demonstrated fewer changes of cytoplasmic organelles compared with the diabetes group. Also indicates the presence of Sertoli cells with irregular morphology adjacent to a well-defined basal lamina and with cytoplasm comprising mitochondria and nuclei with deep indentations (Fig. 7c). Spermatogenic cells contained multiple mitochondria and normal-appearing rough endoplasmic reticulum was also observed. Nuclei were spherical with patchy chromatin content. Well-defined cytoplasmic and nuclear structures were observed in the round spermatids (Fig. 8g,h,i). Leydig cells had an almost normal appearance with multiple lipid droplets, mitochondria, and a large nucleus comprising a thick rim of heterochromatin adhering to the inner surface of an irregular nuclear envelope (Fig. 9c).

Electron microscopy of tissues from the late MSCs treatment group demonstrated more advanced degenerative changes in testicular tissues compared to the diabetes group. Sertoli cells contained irregularly shaped nuclei and scattered aberrant mitochondria (Fig. 7d). The majority of spermatogenic cells had vacuolated mitochondria. Clusters of heterochromatins on the inner surface of the nuclear envelope were observed in the nuclei of spermatogonia. Spermatids had more extensive changes with cytoplasm containing vacuolated mitochondria and fragmented rough endoplasmic reticulum (Fig. 8j,k,l). Leydig cells contained lipid droplets, broken rough endoplasmic reticulum, and clumps of highly condensed chromatin on the internal surface of the Nuclear envelop (Fig. 9D).
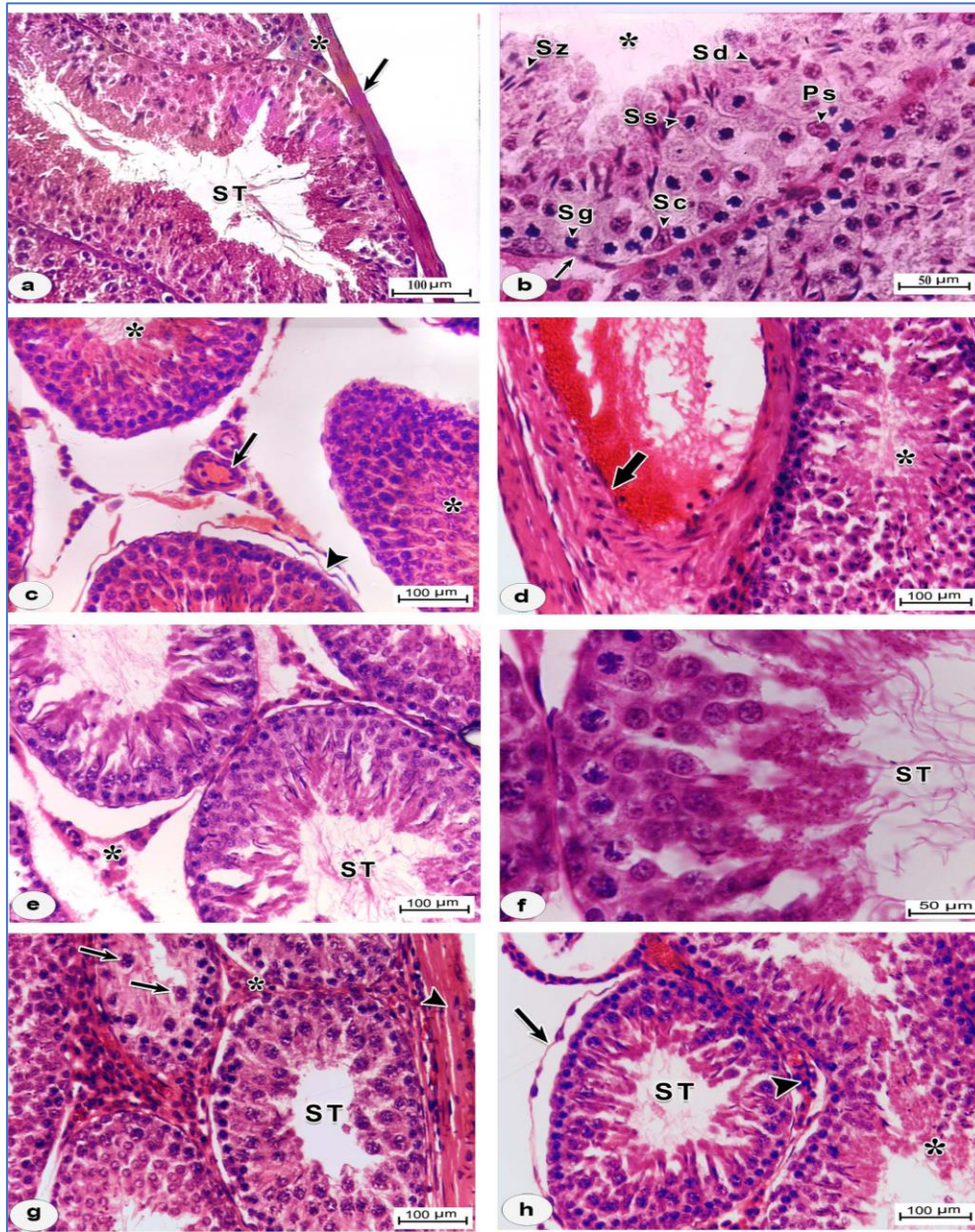
**Fig. 6.** Photomicrographs of testicular tissue sections in the male rats of control group (a,b), diabetes group (c,d), early MSCs treatment group (e,f), and late MSCs treatment group (g,h). **(a)** Tunica albuginea (arrow), seminiferous tubules (ST), and interstitial tissue (*). **(b)** Successive stages of spermatogenesis: spermatogonia (Sg), primary spermatocytes (Ps), secondary spermatocytes (Ss), spermatids (Sd), and spermatozoa (Sz). Sertoli cells (Sc) were observed close to the basal lamina. Leydig cells (*) were also observed. **(c)** Absence of spermatozoa in the lumen of some seminiferous tubules (*), congestion and thickening of endothelial lining of blood vessels (arrow), and hemorrhagic edema in addition to detachment of the basal lamina of some seminiferous tubules ST (arrowhead). **(d)** Sloughing of seminiferous tubules with complete absence of spermatogonia in some tubules (*) with blood vessel congestion and thickening of the endothelial lining (arrow). **(e)** Successive stages of spermatogenesis in most seminiferous tubules (ST) with widening of the interstitial space and hypoplasia of Leydig cells(*). **(f)** Seminiferous tubules (ST) had restoration of normal architecture. **(g)** Extreme degenerative changes and azoospermia in some seminiferous tubules (ST), with multinucleated giant cells (arrows) and thickening of the tunica albuginea (arrowhead) and interstitial cells (*). **(h)** Sloughing of seminiferous tubules(arrow) and complete absence of spermatogonia in the lumen of some ST (*) with intertubular edema (arrowhead).

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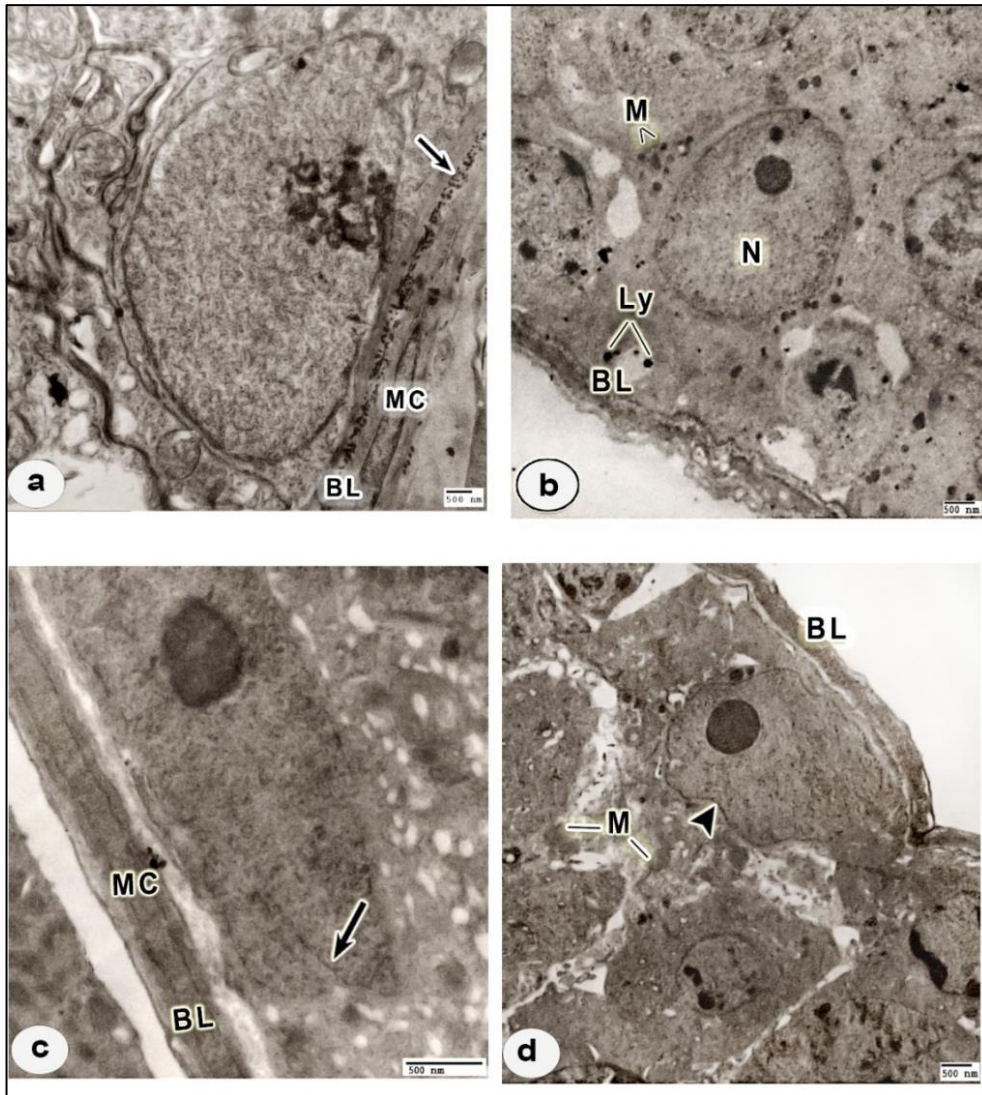


**Fig. 7.** Electron micrographs of boundary tissues in testicular sections from the control group of male rats (a), diabetes group (b), early MSCs treatment group (c), and late MSCs treatment group (d). **(a)** Inner and outer layers of boundary tissue comprising collagenous fibers (arrow), myoid cells (MC), and Sertoli cells on the basal lamina (BL). **(b)** Sertoli cell observed distant from boundary tissue with an irregularly shaped oval nucleus (N). The inner and outer layers of boundary tissue (BL) comprised irregular collagenous fibers. Sertoli cell cytoplasm contained vacuolated mitochondria (M) and lysosomes (Ly). **(c):** Boundary tissue consisting of inner and outer layers of collagenous fibers and myoid cells (MC) with a Sertoli cell observed at the basal lamina (BL) with a deep indentation (arrow). **(d)** The basal lamina (BL) with irregular inner and outer collagenous fibers and a Sertoli cell containing mitochondria (M) and an irregular nuclear envelope (arrowhead).
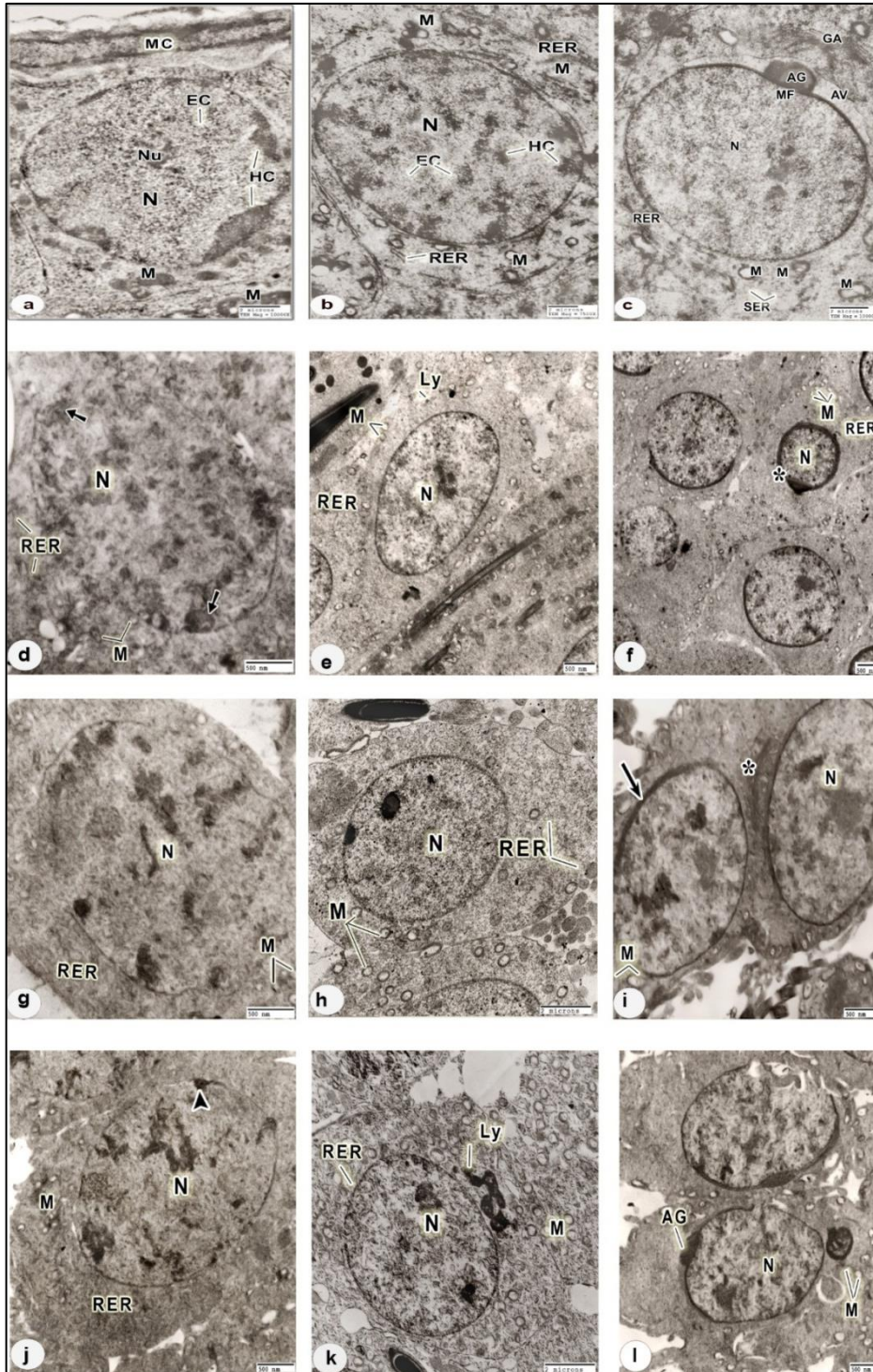
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**Fig. 8.**

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**Fig. 8.** Electron micrography of testicular tissues of male rats showing spermatogonia, primary spermatocytes, and spermatids in the control group (a,b,c), diabetes group (d,e,f), early MSCs treatment group (g,h,i), and late MSCs treatment group (j,k,l). **(a)** Myoid cell (MC) in peritubular tissue with an elongated nucleus (N). The nucleus of a spermatogonium is observed containing heterochromatin (HC) and euchromatin (EC). In addition, the nucleolus (Nu) is clearly seen within the nucleus. The cytoplasm contains mitochondria (M). **(b)** Primary spermatocyte with a spherical nucleus (N) and homogeneous distribution of euchromatin (EC) with lighter condensation and darker heterochromatin (HC). The cytoplasm contains mitochondria (M) and rough endoplasmic reticulum (RER). **(c)** Cephalic cap of a spermatid with an acrosomal vesicle (AV) encapsulating the apical nuclear pole (N). At the marginal fossa (MF) of the nucleus, an acrosomal granule (AG) is observed. A newly formed acrosome is observed within the Golgi apparatus cisternae and vesicles (GA). Smooth endoplasmic reticulum (SER), rough endoplasmic reticulum (RER), and vacuolated mitochondria (M) are observed in the cytoplasm. **(d)**. Spermatogonium with vacuolated mitochondria (M), aberrant rough endoplasmic reticulum (RER) and a spherical nucleus (N). Clumps of heterochromatin (arrows) were observed adhered to the inner surface of the nuclear envelope. **(e)** Primary spermatocyte with highly aberrant mitochondria (M) and fragmented rough endoplasmic reticulum (RER). **(f)** Aberrant oval and round spermatids containing nuclei (N), a dark acrosomal head (*), vacuolated mitochondria (M), and fragmented rough endoplasmic reticulum (RER). **(g** Spermatogonium with a dense spherical nucleus (N), mitochondria (M), and rough endoplasmic reticulum (RER). **(h):** Primary spermatocyte with a large spherical nucleus (N). The cytoplasm contained vacuolated mitochondria (M) and scattered rough endoplasmic reticulum (RER). **(i)** Early spermatids with spherical nuclei (N) and acrosomal vesicles (arrow). The remains of the Golgi apparatus (*) and vacuolated mitochondria (M) are also observed in the cytoplasm. **(j)** Spermatogonium with a spherical nucleus (N) containing clumps of heterochromatin (arrowhead) adherent to the inner membrane of the nuclear envelope, mitochondria (M), and degenerated rough endoplasmic reticulum (RER). **(k)** Primary spermatocyte with a large spherical nucleus (N). The cytoplasm contains vacuolated mitochondria (M), aberrant rough endoplasmic reticulum (RER), and lysosomes (Ly). **(l)** Oval spermatids with spherical nucleus (N), acrosomal granules (AG), and vacuolated mitochondria (M).
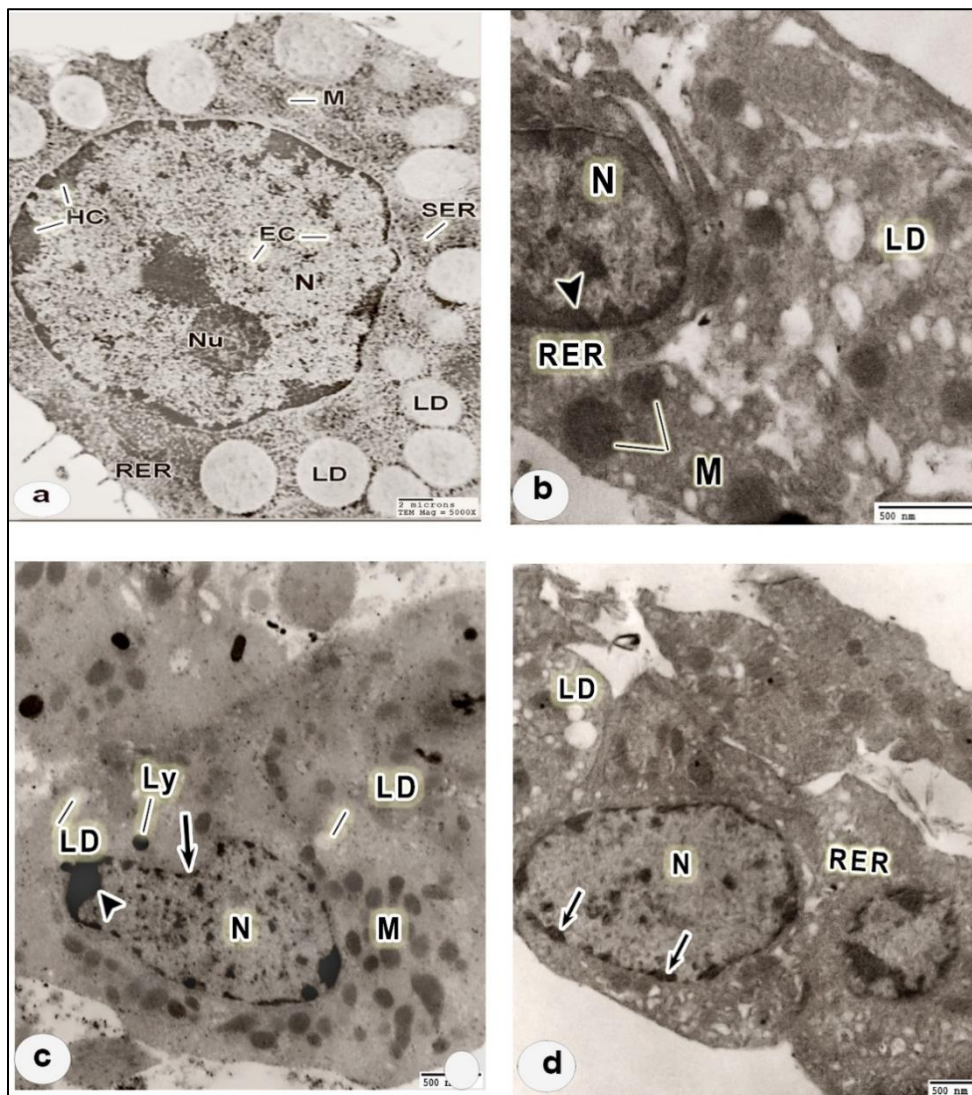
**Fig. 9.** Electron micrographs of Leydig cells in testicular tissues from the male rats in control group (a), diabetes group (b), early MSCs treatment group (c), and late MSCs treatment group (d). **(a)** Leydig cell with a spherical nucleus (N). The nucleolus (Nu) is clearly observed within the nuclear envelope. In addition, two types of chromatin were observed; peripheral heterochromatin (HC) adhering to the inner surface of the nuclear envelope and dispersed euchromatin (EC). Lipid droplets (LD), rough endoplasmic reticulum (RER), smooth endoplasmic reticulum (SER), and mitochondria (M) were observed in the cytoplasm. **(b)** Leydig cell containing numerous lipid droplets (LD), and a fragmented rough endoplasmic reticulum (RER) . Mitochondria (M) had increased electron density. The nucleus (N) contained clumps of heterochromatin on the inner surface of the nuclear envelope (arrowhead). **(c)** Leydig cell with few lipid droplets (LD), mitochondria (M), and lysosomes (Ly). The nucleus (N) contained clumps of heterochromatin (arrowhead) adherent to the inner surface of an irregular nuclear envelope (arrow). **(d)** Leydig cell with numerous lipid droplets (LD) and a fragmented rough endoplasmic reticulum (RER). The nucleus (N) contained heterochromatin clumps on the inner surface of the nuclear envelope (arrows).

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## DISCUSSION

The present study used the STZ-induced rat model of diabetes as STZ, a diabetogenic agent, which has been widely used to evaluate the adverse effects of DM on spermatogenesis and testicular steroid-genesis (Sampannang *et al*., 2018)**.** DM can induce testicular dysfunction due to hyperglycemia. Insulin deficiency is the commonest cause of hyperglycemia which has adverse effects on the body (Ding *et al*., 2015). The current study MSCs had been used in alleviating testicular dysfunction in prepubescent male rats with diabetes. Millman and Pagliuca (2017) and Yousef *et al*. (2022) indicated that MSCs can be used for cell replacement therapy. The intravenous injection of BM-MSCs in the STZ-induced rat model of diabetes restored β-cell function and restored glucose metabolism homeostasis (Yousef *et al*., 2022). In the present study, biochemical analyses demonstrated that STZ-treated rats had reduced serum testosterone, LH, and FSH levels. Reduced insulin secretion affects gonadotropin (FSH and LH) production, which are required for increasing androgen secretion. Consequently, lower levels of circulating FSH and LH lead to decrease testosterone production.

DM has been shown to interrupt the hypothalamic-pituitary-gonadal (HPG) axis and decrease FSH and LH responses to gonadotropin releasing hormone (GnRH) (Salah *et al*., 2022). Diabetes-related changes including increased oxidative stress, lipid peroxidation, proinflammatory cytokine levels in testicular tissues, and may also contribute to decreased in serum testosterone levels (Bahey *et al*., 2014).

The present findings demonstrated that diabetes induced during the early postnatal period in rats lowers circulating testosterone and suppresses the physiological effects of androgen action, confirming the antiandrogenic effect of diabetes in rats. As prepuberty is a critical period for the development of fertility, damage to testicular cells or the HPG axis during this time may reduce fertility in adult rats (Moody *et al*., 2013).

Testis is particularly vulnerable to oxidative stress due to high polyunsaturated fatty acid content and low antioxidant efficiency (Beytur *et al*., 2012). In the present study, STZ-induced hyperglycemia caused markedly increased levels of oxidative stress in the testis, as demonstrated by increased levels of TBARS. In addition, STZ treatment reduced the activities of antioxidant enzymes including SOD, CAT, GSH, and GPx in the testicular tissue of prepubescent diabetic rats. These metabolic disturbances were associated with increased serum levels of the proinflammatory cytokines TNF-α and IL-1β, which may have increased levels of apoptosis. These findings corroborate the results of Long *et al*. (2015) and Zha *et al*. ( 2018) who posited that hyperglycemia may cause apoptosis and oxidative stress through increased production of reactive oxygen species (ROS) which lead to loss of reproductive function by inducing inflammation and damage to the testis in diabetic rats.

The results of the biochemical analyses in the present study were confirmed by histological and ultrastructural observations. In the present study, STZ-treated prepubescent male rats had a range of pathological changes in the developing testis. Two types of cells (spermatogonia and Sertoli cells) were found in the seminiferous tubules of prepubescent rat testis (Oatley and Brinster, 2012). At puberty, Sertoli cells

form inter-Sertoli cell junctional complexes to create a blood-testis barrier which it was considered critical for spermatogenesis and creating an environment suitable for germ cells meiosis (Elkin *et al.*, 2010). Further, Sertoli cells have been shown to contribute to the development of testicular vasculature by inducing differentiation of peritubular myoid cells and fetal Leydig cells that support spermatogenesis within the seminiferous tubule through the action of FSH and testosterone (Karl and Capel, 1998). Accordingly, damage to these cells is likely to have deleterious effects on the whole testicular tissue. In the present study, STZ-treated rats had impaired spermatogenic function with alterations observed in all germ cell types in the seminiferous tubules, with a proportion of seminiferous tubules found to be sperm-free while others had arrest of spermatogenesis. In addition, multiple vascular alterations (thrombosis and interstitial tissue hemorrhage) were also observed. Meistrich (1986) reported that any substance that disrupts the vascular supply causes an anoxic reaction in the dependent organ.

The findings of the present study are consistent with previous studies (Moody *et al.*, 2013; Ricci *et al.*, 2009) reporting that the development of diabetes during prepubescence increases the proportion of animals with severely damaged tubules accompanied by spermatogenetic arrest and the absence of sperm in a high proportion of seminiferous tubules.

Ultrastructural analyses in the present study demonstrated that exposure to STZ induced severe changes in germ cells, Sertoli cells, and Leydig cells. The mechanisms underlying the effects of STZ on testicular function are likely mediated by direct inhibitory effects on the testes or indirect effects on the hypothlamo-pituitary axis given the findings of the above studies. As Leydig cells are the predominant source of testosterone, reduced Leydig cell function

may lead to male infertility (Ellis, 2011). Diabetes-induced oxidative stress is known to affect Leydig cell function and decrease testosterone levels and causes alterations in the seminiferous epithelium of diabetic rats (Ricci *et al.*, 2009).

In the present study, serum levels of testosterone, FSH, and LH levels were higher in the diabetes group compared to the early MSC treatment group. In addition, MSC treatment significantly reduced the deleterious histological and ultrastructural changes observed in the testicular tissues of rats in the diabetes group, with decreased ROS (TBARS) and increased activity of antioxidant enzymes (SOD, CAT, GSH, and GPx). MSC therapy also reduced apoptosis and inflammation in testicular tissues with reducing TNF-α and IL-1β levels. Moreover, early treatment with BM-MSCs reduced atrophy of seminiferous tubules, possibly due to the free radical scavenger and anti-apoptotic properties of MSCs. Moreover, the histological examinations and electron microscopy demonstrated Sertoli cells and germ cells with normal appearance in the late MSC treatment group and with spermatozoa within the lumen of the seminal duct.

The recovery of testicular injury in diabetic rats treated with BM-MSCs is consistent with the findings of Zhankina *et al.* (2021) who evaluated the therapeutic benefits of MSCs in animal models of non-obstructive azoospermia (NOA) induced by chemical or surgical agents. These experiments demonstrated that MSCs can be successfully allotransplanted or xeno-transplanted into seminiferous tubules. Furthermore, the authors reported that exosomes released by MSCs may promote spermatogenesis in animal models of infertility. Further, embryonic stem cells may be found in the basal layer of the testicular seminiferous tubules and give rise to progenitor cells (Chikhovskaya *et al.*, 2012). These cells have been shown to

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survive chemotherapy and induce germinal cell differentiation (Bhartiya, 2018). The interaction between these cells and transplanted MSCs is believed to have a key role in restoring fertility (Zhankina *et al*., 2021). MSCs may secrete factors that promote the differentiation of progenitor cells present in testicular tissue, thereby restoring tissue function as MSCs can be transplanted in undifferentiated stages to allow differentiation into more specialized cells that secrete factors and perform trophic roles (Maqsood *et al*., 2020). Yousef *et al*. (2022) demonstrated that by regenerating β cells, the pancreatic damage can be repaired, ensuring that the testicular tissue didn't experience an excess of the DM's negative effects.

In the current study, rats in the late MSCs treatment group had no significant improvements in biochemical, histological, or ultrastructural analyses of testicular tissues. This may be attributable to the administration of MSCs after a longer duration of injury. The administration of stem cells at an early stage of diabetes may increase stem cell function compared to its administration at a later stage.

The current results demonstrated that the testicular dysfunction observed can be prepubescent rats treated with STZ is attributable to cumulative oxidative disturbances at the subcellular level that may have profound consequences on puberty and fertility.

## Conclusions

The findings of the present study demonstrate that early systemic administration of bone marrow mesenchymal stem cells (BM-MSCs) to prepubescent rats protects against testicular injury and may restore testicular function in a model of STZ-induced diabetes. The protective effects of BM-MSCs may be mediated by reducing STZ-induced oxidative stress, inflammation, and apoptosis.

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# Ameliorating effects of mesenchymal stem cells on testicular injury in a prepubescent male rat model of streptozotocin-induced diabetes

**التاثيرات المحسنة المحتملة  للخلايا الجذعية الوسيطة لنخاع العظم على خصية الجرذان المهقاء المصابة بمرض السكري المستحث بمادة الاستربتوزوتوسين قبل البلوغ**

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**المستخلص**

تساهم المستويات المتزايدة من الإجهاد التأكسدي والالتهاب والموت المبرمج للخلايا نتيجة الاصابة بداء السكري في ضعف خلايا الخصية. وفقًا لذلك، تهدف الدراسة الحالية إلى تقييم التأثيرات الوقائية المحتملة للحقن بالخلايا الجذعية الوسيطة لنخاع العظم (BM-MSCs) على إصابة الخصية في ذكور الجرذان المصابة بداء السكري المستحث بمادة الاستربتوزوتوسين قبل البلوغ. استخدم في هذه الدراسة اربع وعشرون من ذكور الجرذان المهقاء البالغة من العمر أربعة أسابيع، والتى تم تقسيمها بالتساوي الي إلى اربع مجموعات: المجموعة الضابطه (تم حقنها بمحلول ملحي فقط)، مجموعة داء السكري (تم حقنها بمادة الاستربتوزوتوسين)، مجموعة داء السكري المعالجة بجرعة واحدة من BM-MSCs قيمتها $2×10^6$ بعد أسبوع من العلاج بمادة الاستربتوزوتوسين (المجموعة المعالجة بالخلايا الجذعية فى فترة عمرية مبكرة)، مجموعة داء السكري المعالجة بجرعة واحدة من BM-MSCs قيمتها $2×10^6$ بعد اربع اسابيع من العلاج بمادة الاستربتوزوتوسين (المجموعة المعالجة بالخلايا الجذعية فى فترة عمرية متأخرة). بعد سبعة أيام من الحقن بالخلايا الجذعية، تم الحصول على عينات من المصل وأنسجة الخصية لإجراء التحليلات الكيموحيوية والنسيجية والتركيبية الدقيقة. تم تسجيل انخفاض ذو دلالة احصائية في محتوى مصل الجرذان المعالجة بمادة الاستربتوزوتوسين من هرمونات التستوستيرون، الهرمون المنشط للحوصلة، والهرمون المنشط للجسم الأصفر مقارنة بالمجموعة الضابطة.  لوحظ من النتائج انخفاض ملحوظ في مستوى الجلوتاثيون، ونشاط كل من السوبر أكسيدً ديسميوتيز، الكاتاليز، الجلوتاثيون بيروكسيديز في أنسجة الخصى للجرذان المعالجة بمادة الاستربتوزوتوسين مقارنةً بالمجموعة الضابطة. في المقابل تم تسجيل زيادة ذات دلالة احصائية في مستويات انسجة خصى الجران المعالجة بمادة الاستربتوزوتوسين من القدرة المؤكسدة الكلية، حمض الثيوباربتيريك القابل للتفاعل، ومستويات عامل الفا  لتنكرز الأورام $α$-TNF، والإنترلوكين- β1 مقارنةً بمثيلاتها في المجموعة الضابطة. كما أوضح الفحص النسيجى لأنسجة الخصي من الجرذان المعالجة بمادة الاستربتوزوتوسين تغيرات واضحة فى البناء النسيجى فى خصى الحيوانات المعالجة بالعقار، تضمنت مظاهر التهدم حدوث خلل فى ترتيب وتنظيم الطلائية الجرثومية مع النقص الواضح فى سمك طبقاتها فى بعض الانيبيبات المنوية وامتد ذلك الى غياب الحيوانات المنوية فى بعض من الانيبيبات المنوية مع ظهور انيبيبات منوية لم يكتمل بها عملية تكوين الحيوانات المنوية. وفيما يتعلق بالنسيج البينى فإن اهم التغيرات التى لوحظت كانت نزيف دموى وإحتقان بعض الاوعية الدموية. ومن الناحية التركيبية الدقيقة فقد أظهرت الخلايا المنوية تغيرات فى العضيات المنتشرة بالسيتوبلازم والذى تمثل فى تحلل للميتوكندريا وتمدد لعناصر الشبكة الاندوبلازمية الخشنة. كما أظهرت بعض خلايا سرتولى انبعاج فى أغلفاتها النووية. وتشير نتائج الدراسة الحالية إلى أن الحقن الوريدي المبكر بالخلايا الجذعية الوسيطة لنخاع العظم خفف معظم تأثيرات مرض السكري المستحث بمادة الاستربتوزوتوسين في أنسجة الخصية من الفئران قبل البلوغ.